

Production of Apx Toxins by Field Strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*

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The three Apx toxins of *Actinobacillus pleuropneumoniae* have potential value for use in vaccines and diagnostic tests which will be species specific instead of serotype specific, provided that the Apx toxins are species specific and all field strains produce these toxins. We examined 114 *A. pleuropneumoniae* field strains and found that they secreted either ApxI, ApxII, ApxI and ApxII, or ApxII and ApxIII and secreted no other cytolytic activities. However, proteins similar to ApxI and ApxII were also produced by *Actinobacillus suis*.

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease which is spread worldwide and causes substantial economic losses to the pig industry (23). Two biotypes of *A. pleuropneumoniae* are recognized. Biotype 1 is NAD dependent and biotype 2 is NAD independent. So far, 14 serotypes have been recognized, 12 within biotype 1 (20) and 2 within biotype 2 (7). The prevalence of serotypes varies widely by geographic region.

Reference strains of all serotypes of biotype 1 secrete cytolytic toxins that belong to the RTX family (8, 12, 14, 24). Three toxins named ApxI, ApxII, and ApxIII are known (8). ApxI is strongly hemolytic and strongly cytotoxic, ApxII is weakly hemolytic and moderately cytotoxic, and ApxIII is nonhemolytic but strongly cytotoxic. The reference strains of biotype 1, serotypes 1, 5, 9, and 11, secrete ApxI and ApxII; serotypes 2, 3, 4, 6, and 8 secrete ApxII and ApxIII; serotypes 7 and 12 secrete ApxII; and serotype 10 secretes ApxI (14). Reference strains of biotype 2 were not investigated. In addition, it was shown recently that *Actinobacillus suis*, an opportunistic pathogen of swine, produces a toxin which is identical to ApxII (2).

In countries where many serotypes of *A. pleuropneumoniae* are prevalent, vaccines and diagnostic tests that are species specific instead of serotype specific are needed. The Apx toxins have potential value for use in such vaccines and tests, provided that the toxins are species specific and that all field strains produce these, and no other, cytolytic toxins. Only little is known about toxin production of field strains. The few studies that have been published were conducted before the three Apx toxins had been identified (6, 15, 21). We have monoclonal antibodies (MAbs) and neutralizing antisera against the toxins, and with these tools we studied in detail the production of the Apx toxins by field strains.

Colleagues from 10 different countries kindly donated 114 *A. pleuropneumoniae* strains. These strains represented biotypes 1 and 2 and all serotypes except serotype 4 of biotype 1. The origin and the distribution of these strains among the serotypes are listed in Table 1.

The identities and the serotypes of the strains were verified as described earlier (13). When we determined a serotype other than that determined by the donor of a strain, the strain

was sent to J. Nicolet, University of Bern, Bern, Switzerland, or to R. Nielsen, State Veterinary Serum Laboratory, Copenhagen, Denmark, for definite serotyping.

In addition to the *A. pleuropneumoniae* strains, we tested *A. suis* type strain NCTC 10840 and four Dutch *A. suis* field strains. The strains were classified phenotypically (3) and differed from *A. pleuropneumoniae* biotype 2 in a positive test result for esculin, salicin, trehalose, and growth on MacConkey agar and a negative test result for mannitol.

Toxin-containing culture filtrates were obtained as described by Frey and Nicolet (9), and each filtrate was tested for the presence of Apx toxins with each of the MAbs CVI-Ap1y 2.2, 9.1, 9.2, 9.3, and 9.4 (14) in a dot blot assay according to the recommendations of the manufacturer of the dot blot apparatus (Bio-Rad, Richmond, Calif.). The molecular masses of the proteins that reacted with the MAbs were determined by Western blot (immunoblot) analysis (14) of 10-fold-concentrated culture filtrates with a mixture of the five MAbs. Culture filtrates of the reference strains of biotype 1, serotypes 8 and 11, were used as references.

Each filtrate was further tested for hemolytic (9) and cytotoxic (15) activities. A filtrate was defined as strongly hemolytic when it totally hemolyzed erythrocytes and as weakly hemolytic when it only partially hemolyzed erythrocytes and hemolysis could not be read with the naked eye. A filtrate was defined as strongly cytotoxic when it had a titer of ≥ 64 and as moderately cytotoxic when it had a titer of ≤ 32 .

Filtrates of all strains but one belonging to biotype 1, serotypes 1, 5, 9, and 11, reacted in the dot blot assay with all MAbs against ApxI and ApxII, and Western blot analysis revealed two proteins that comigrated with ApxI and ApxII. All filtrates were strongly hemolytic and strongly cytotoxic. We concluded that these strains produced ApxI and ApxII (Table 2). The one exception was a serotype 9 strain from Australia, which produced ApxII only (Table 2).

Filtrates of all strains but one belonging to biotype 1, serotypes 2, 3, 6, and 8, reacted in the dot blot assay with all MAbs against ApxII and ApxIII, and Western blot analysis revealed two proteins that comigrated with ApxII and ApxIII. All of these filtrates were weakly hemolytic and strongly cytotoxic. We concluded that these strains produced ApxII and ApxIII (Table 2). The one exception was a serotype 2 strain from Australia, which produced ApxII only (Table 2).

Filtrates of all strains but two belonging to biotype 1, serotypes 7 and 12, and of all strains belonging to biotype 2, serotypes 1 and 2, reacted in the dot blot assay with the MAbs

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TABLE 1. Origin and distribution of *A. pleuropneumoniae* strains among the serotypes

Origin	No. of strains per serotype:													
	1 ^a	2 ^a	3	5a	5b	6	7	8	9	10	11	12	1 ^b	2 ^b
Australia	1	1	2	1			1		1					
Belgium		5	3		1	1			1	1				4
Canada	10			5										
Denmark						4				2		3		
France		1	1						2		1			
Hungary													1	1
Ireland			1											
The Netherlands		8	10			2	9	5	4		8			5
New Zealand							7							
United States				1										

^a Serotypes 1 and 2 of biotype 1.^b Serotypes 1 and 2 of biotype 2.

against ApxII only, and Western blot analysis revealed one protein that comigrated with ApxII. The filtrates were weakly hemolytic and moderately cytotoxic. We concluded that these strains produced ApxII (Table 2). The two exceptions were two biotype 1, serotype 7, strains from The Netherlands, which produced ApxII and ApxIII (Table 2).

Filtrates of all strains belonging to biotype 1, serotype 10, reacted in the dot blot assay with the MABs against ApxI only, and Western blot analysis revealed one protein that comigrated with ApxI. These filtrates were strongly hemolytic and strongly cytotoxic. We concluded that these strains produced ApxI (Table 2).

Filtrates of the *A. suis* strains reacted in the dot blot assay with all MABs against ApxI and ApxII, and Western blot analysis revealed two proteins that comigrated with ApxI and ApxII (Fig. 1); the filtrates were strongly hemolytic and strongly cytotoxic. We concluded that these strains produced proteins similar to ApxI and ApxII.

To determine whether the field strains produced cytolytic proteins other than those produced by the reference strains of *A. pleuropneumoniae*, each filtrate was tested in a cytotoxin neutralization assay with a serum against crude Apx toxins of *A. pleuropneumoniae* reference strains (15). Filtrates of strains producing only ApxI were tested with an antiserum against ApxI of biotype 1, serotype 10; filtrates of strains producing only ApxII were tested with an antiserum against ApxII of biotype 1, serotype 7; filtrates of strains producing ApxI and ApxII were tested with the antiserum against ApxI and ApxII of biotype 1, serotype 11; and filtrates of strains producing ApxII and ApxIII were tested with the antiserum against ApxII and ApxIII of biotype 1, serotype 8. The cytolytic activities of the filtrates of all strains, including those of *A. suis*, were completely neutralized, indicating that the strains produced no

TABLE 2. *A. pleuropneumoniae* field strains of different serotypes that produce the Apx toxins

Apx toxin	No. of strains of serotype:													
	1 ^a	5a	5b	9	11	2 ^a	3	6	8	7	12	1 ^b	2 ^b	10
I														3
II				1		1				15	3	1	10	
I + II	11	7	1	7	9									
II + III						14	17	7	5	2				

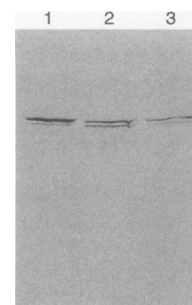
^a Serotypes 1 and 2 of biotype 1.^b Serotypes 1 and 2 of biotype 2.

FIG. 1. Western blot of culture filtrates probed with a mixture of MABs CVI-ApI 9.2, 9.3, and 9.4. Lane 1, culture filtrate of the reference strain of *A. pleuropneumoniae* serotype 11 which produces ApxI (110 kDa) and ApxII (103 kDa); lane 2, culture filtrate of *A. suis* NCTC 10840; lane 3, culture filtrate of an *A. suis* field strain.

cytolytic activity in addition to that produced by the serotype reference strains of *A. pleuropneumoniae*. This cytolytic activity of the above reference strains was due to the Apx toxins only, because an antiserum against purified recombinant ApxI, -II, and -III (12, 24) completely neutralized the cytolytic activity of the above reference strains.

Infections with *A. pleuropneumoniae* are usually serologically diagnosed. The complement fixation test (18) or enzyme-linked immunosorbent assays (ELISAs) with capsular antigens (1, 19) are most widely used. These assays are serotype specific, which is disadvantageous in countries where many serotypes are prevalent. A diagnostic test which is species specific instead of serotype specific is preferred. In this study, we found that *A. pleuropneumoniae* field strains produce either ApxI, ApxII, ApxI and ApxII, or ApxII and ApxIII. Thus, the Apx toxins have potential value as antigens in a diagnostic test. Cross-reactions with RTX toxins of other bacteria can be expected (6) but may be overcome by use of specific MABs in blocking ELISAs. ApxII would be the antigen of first choice because it is produced by all *A. pleuropneumoniae* strains except those of serotype 10 and because the ApxII toxins of all serotype reference strains are similar (11). However, *A. suis* produces an RTX toxin which is identical to ApxII even at the amino acid level (2). In this study, we confirmed that *A. suis* strains produce a toxin similar to ApxII, but we also found that *A. suis* produces a toxin similar to ApxI. We therefore assume that serological tests based on ApxI, ApxII, or both will detect infections not only with *A. pleuropneumoniae* but also with *A. suis*.

Two indirect ELISAs based on Apx toxins have been reported. One ELISA uses a 104-kDa hemolysin of serotype 1 (5), and the second ELISA uses a 110-kDa hemolysin of serotype 5 (17). Although the molecular masses suggest that the first ELISA uses ApxII and the second ELISA uses ApxI, this is not certain. Both serotypes 1 and 5 produce ApxI and ApxII, and these toxins are not separated by standard purification methods. Furthermore, they are not easily separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and thus the estimates of the molecular masses of the hemolysins may have been inaccurate. In agreement with our expectations, the ELISA with Apx of serotype 1 reacted positively with sera from pigs of a herd with clinical *A. suis* disease. In contrast to our expectations, the ELISA based on Apx of serotype 5 was specific for *A. pleuropneumoniae*. Possibly, the pigs that were used to evaluate the latter ELISA did not carry *A. suis*.

Still, we think that it may be possible to develop one or more

ELISAs based on Apx toxins that can be used for diagnosis of porcine pleuropneumonia for several reasons. First, we know too little about the prevalence of *A. suis* in pig populations; second, we do not know to what extent ApxI is similar to the corresponding toxin of *A. suis*; and third, we do not know whether pigs that only carry *A. suis* produce antibodies against its cytolytic toxins.

The Apx toxins are essential components in protective vaccines (10). Whether a vaccine of the toxins alone will be sufficient for protection is not clear yet; data published so far on vaccination with purified Apx toxins are inconclusive (4, 22). In this study, we found no cytolytic proteins other than ApxI, -II, and -III. Hence, incorporation of ApxI, -II, and -III into a vaccine may protect pigs against challenge with any *A. pleuropneumoniae* strain. This hypothesis is supported by our findings that a mixture of culture filtrates of serotypes 3 and 9 protected pigs against challenge with homologous and heterologous serotypes (16).

In a previous study in which we identified the three cytolytic proteins among the serotype reference strains of *A. pleuropneumoniae*, we reported that the reference strain of serotype 6 produced ApxII only (14). While examining field strains of serotype 6, we found that we had the wrong strain as the reference for serotype 6. Consequently, the data we have published on serotype 6 are incorrect (13–15). Strain Femo, which is the reference strain for serotype 6, produces ApxII and ApxIII. The strain we used as a reference for serotype 6 may represent a new serotype. Antiserum raised against this strain does not agglutinate any other serotype reference strain, and the strain does not agglutinate in antisera raised against the other *A. pleuropneumoniae* serotypes.

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